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ATTACHMENTS TO AMENDMENT

(1) Hard *et al.*, "Solution Structure of the Glucocorticoid Receptor DNA-Binding Domain," *Science 249*:157-160 (1990)

(2) Liden *et al.*, "A New Function for the C-terminal Zinc Finger of the Glucocorticoid Receptor," *J. Biol. Chem. 72(34)*:21467-21472 (1997)

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Solution Structure of the Glucocorticoid Receptor DNA-Binding Domain

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The three-dimensional structure of the DNA-binding domain (DBD) of the glucocorticoid receptor has been determined by nuclear magnetic resonance spectroscopy and distance geometry. The structure of a 71-residue protein fragment containing two "zinc finger" domains is based on a large set of proton-proton distances derived from nuclear Overhauser enhancement spectra, hydrogen bonds in previously identified secondary structure elements, and coordination of two zinc atoms by conserved cysteine residues. The DBD is found to consist of a globular body from which the finger regions extend. A model of the dimeric complex between the DBD and the glucocorticoid response element is proposed. The model is consistent with previous results indicating that specific amino acid residues of the DBD are involved in protein-DNA and protein-protein interactions.

THE GLUCOCORTICOID RECEPTOR belongs to a family of ligand-inducible nuclear transcription factors that include the steroid hormone, thyroid hormone, retinoic acid, and vitamin D_3 receptors. All members of this superfamily contain a highly conserved DNA-binding domain that consists of about 70 residues and mediates specific binding to hormone response elements on DNA (*1*). Protein fragments containing the glucocorticoid receptor DBD expressed in *Escherichia coli* exhibit sequence-specific binding to glucocorticoid response elements (GREs) (*2*, *3*). These protein fragments contain two zinc atoms, tetrahedrally coordinated by conserved cysteine residues, that are required for proper folding and DNA binding (*2*). The presence of zinc-binding domains is reminiscent of the "zinc finger" motif found in *Xenopus* TFIIIA (*4*), as well as similar domains found in retroviral nucleic acid binding proteins (*5*). However, the hormone receptor zinc-coordinating regions are not homologous to these other zinc fingers, suggesting that the DNA-binding domain of the steroid and thyroid hormone receptors constitutes a distinctive structural motif (*6*).

We have studied two protein fragments containing the glucocorticoid receptor DBD using two-dimensional nuclear magnetic resonance (2D NMR) and distance geometry (DG). These fragments contain 93 and 115 residues, respectively, with a common sequence encompassing the Cys^{440} to Ile^{519} and Cys^{421} to Ile^{500} segments of the rat and human glucocorticoid receptors, respectively (*7*, *8*). The structural studies focus on the 71-residue segment Cys^{440} to Arg^{510}, which includes the two zinc-coordinating finger regions (Fig. 1). Sequence-specific assignments of more than 90% of all observable ^1H resonances within this segment were obtained with the use of 2D double quantum filtered correlated spectroscopy (DQF-COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), and nuclear Overhauser enhancement spectroscopy (NOESY). The resonance assignments (*9*) were carried out with the use of well-established procedures (*10*, *11*).

Several elements of secondary structure within the Cys^{440} to Arg^{510} segment could be identified based on characteristic patterns of NOE connectivities (*10*). These elements include two α-helical regions encompassing Ser^{459} to Glu^{469} and Pro^{493} to Gly^{504}, a type I reverse turn between residues Arg^{479} to Cys^{482}, a type II reverse turn between residues Leu^{475} to Gly^{478}, a short stretch of antiparallel β sheet involving residues Cys^{440} and Leu^{441} and Leu^{455} to Cys^{457}, as well as

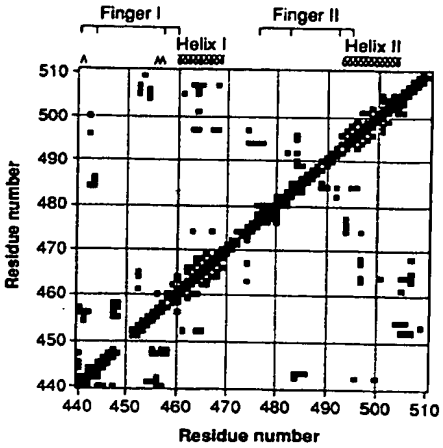

Fig. 2. Diagonal plot indicating residues between which NOEs have been found. Secondary structure elements and zinc coordination within the two finger domains (*19*) has also been indicated.

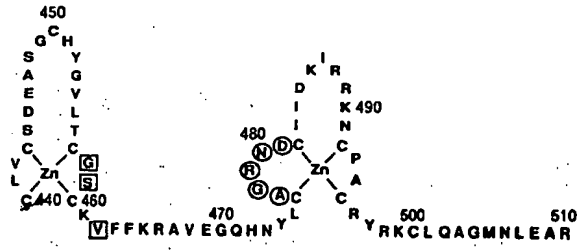

Fig. 1. The segment Cys^{440} to Arg^{510} of the rat glucocorticoid receptor DNA-binding domain (Cys^{421} to Arg^{491} of the human glucocorticoid receptor) for which the three-dimensional structure is presented here (*8*). The boxed residues indicate amino acids that are essential for discrimination between glucocorticoid and oestrogen response elements (GRE and ERE) (*26*). The circled residues indicate the segment that is important for protein-protein interactions in the dimeric DBD-GRE complex (*25*).

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several regions of extended peptide conformation. No evidence for α-helical domains could be found within the two finger regions. The identification of secondary structure elements also revealed that the pattern [finger-helix-extended region] is repeated twice within the DBD. Long-range NOEs resulting from protein folding can be found within and between these distinct regions (Fig. 2). For instance, NOEs are found within the two finger regions, between the two helical domains, between the first finger and the extended carboxyl-terminal region, as well as between the two finger regions.

The three-dimensional protein structure was determined with DG and distance bounds driven dynamics (DDD) (*12*) based on NOE connectivities, hydrogen bonds within identified secondary structure elements, and zinc atom coordination by Cys sulfurs. The structure determination is based on a set of 470 nonredundant NOE connectivities, including 194 sequential, 70 medium-range, and 134 long-range NOEs (*13*). The NOE intensities were converted to upper distance bounds with the use of initial NOE buildup rates obtained from NOESY spectra recorded with different mixing times (*14*) and calibrated versus known intraresidue and sequential proton-proton distances (*15*). The NOEs for which no reliable buildup curves could be obtained were assumed to represent a distance of ≤5 Å. Pseudoatom corrections were added for prochiral methylene groups, methyl groups, Phe and Tyr 2,6 and 3,5 ring protons, and Leu dimethyl groups (the eight Val methyl groups could be stereospecifically assigned) (*16*). Lower distance limits were in all cases taken as the sum of the van der Waals radii of the atoms or groups involved. The NOE constraints were supplemented by 18 amide-carbonyl hydrogen bonds (*17*) within secondary structure elements that could be identified from sequential NOE connectivities (two α helices, two reverse turns, and a short stretch of antiparallel β sheet).

Initial calculations showed that the DG structures could be further refined by including the effect of zinc coordination by conserved Cys residues. Tetrahedral zinc coordination was imposed by enforcing distance bounds of 3.83 ± 0.02 Å (*18*) between the sulfurs of Cys^{440}, Cys^{443}, Cys^{457}, and Cys^{460} in the first finger and Cys^{476}, Cys^{482}, Cys^{492}, and Cys^{495} in the second finger. The zinc coordination scheme within the second finger region has been the subject of some discussion, because this region contains five conserved Cys residues. However, site-directed mutagenesis studies clearly show that the conserved Cys^{500} can be replaced by Ala or Ser without significant loss of glucocorticoid receptor function and that this residue therefore cannot be involved in zinc complexation (*19*). This conclusion is strongly supported by recent studies of mutant DBD fragments in which each of the residues Cys^{492}, Cys^{495}, and Cys^{500} was replaced by Ser, showing that only Cys^{500} can be replaced without obstructing the protein folding (*20*). DG calculations that used tetrahedral zinc coordination within the second finger and excluded either Cys^{492}, Cys^{495}, or Cys^{500} yielded values of the DG error function that significantly favored the latter alternative (*21*). Zinc coordination involving residues Cys^{476}, Cys^{482}, Cys^{492}, and Cys^{495}, but not Cys^{500}, are therefore also consistent with NMR data.

Metric matrix DG was performed with an algorithm based on the original "embed" procedure (*22*), followed by 300 steps of conjugate gradient optimization of the DG constraint function (*12*). All DG structures were further subjected to 500 + 500 steps of DDD at 300 and 1 K, respectively, to obtain a more efficient sampling of "allowed" conformations (*23*). The DG calculations yielded protein structures with an average root-mean-square (rms) difference between peptide backbone atoms of 1.55 Å (Fig. 3). Structures that had been subjected to DDD showed somewhat larger rms differences (2.02 Å). The structure within a region forming a globular protein body (see below) is determined with a higher resolution (backbone rms differences of 1.32 Å after DG embedding and optimization and 1.52 Å after DDD). These data show that

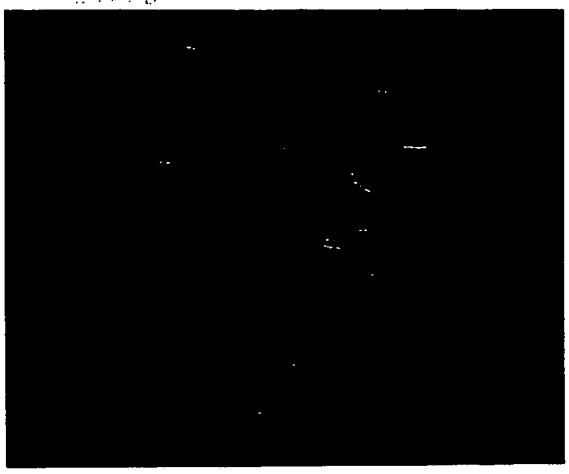

Fig. 3. Polypeptide backbone traces of a family of nine superimposed distance geometry structures for the glucocorticoid receptor DNA-binding domain (residues Cys^{440} to Arg^{510}). The view is approximately the same as in Fig. 4 (left).

Fig. 4. Ribbon model of the glucocorticoid receptor DNA-binding domain with two tetrahedrally coordinated zinc atoms. The view to the left is of the flat surface showing the protein body in the center with the first and second finger regions to the upper right and upper left, respectively. The view to the right is through the first helix (Ser^{459} to Glu^{469}), which is taken as a recognition helix in our model of the DBD-GRE complex (Fig. 5). The colors refer to the segments Cys^{440} to Gly^{470} (red) and Gln^{471} to Arg^{510} (green), including the first and second finger regions, respectively.

the present set of distance constraints is sufficient to describe the backbone folding of the DBD.

The DBD structure is shown in more detail in Fig. 4. The protein fragment has an overall oblate shape with long and short axis diameters of about 35 and 20 Å, respectively. One face is almost completely flat, whereas the other is in the shape of a cone. The two α helices are oriented perpendicular to each other, with hydrophilic surfaces exposed to the solvent. The α-helical regions and the extended peptide regions following each helix form a compact protein body. This body, which is better determined than the finger regions, is almost spherical with a diameter of about 20 Å. Several of the conserved hydrophobic residues, including Tyr^{452}, Phe^{463}, Phe^{464}, Val^{468}, His^{472}, Tyr^{474}, Tyr^{497}, Leu^{501}, Met^{505}, and Leu^{508}, form a hydrophobic core. The two zinc atoms are located outside the body, close to the flat surface, at a distance of about 13 Å from each other. The first finger domain is folded on top of the body and makes several contacts with the two helices and the carboxyl-terminal extended region. The second finger extends out from the body in two loops formed by the segments Ala^{477} to Asp^{481} and Ile^{483} to Asn^{491}. There are also contacts between the two finger domains, mainly involving residues Val^{442} and Cys^{443} and Asp^{485} to Ile^{487}.

The structure of the DBD combined with genetic and biochemical data allow us to propose a model for the DBD-GRE complex. The recombinant DBD fragments studied here bind selectively to GRE DNA sequences that generally appear to consist of two half-site hexamers separated by three base pairs with a consensus sequence of GGTACANNNTGTTCT (where N is any nucleotide). A number of properties of the DBD-GRE complex were considered. First, DBD binds to the two GRE half-sites in a cooperative manner (*24*). Also, substitution of Ala^{477} to Asp^{481} with the corresponding segment of another receptor yields a protein that recognizes the GRE, but binds without cooperativity. Residues within this segment may therefore be determinants for protein-protein interactions in the dimeric complex (*25*). Second, three residues (458, 459, and 462) near the first zinc coordination site discriminate between glucocorticoid and estrogen response elements (*26*). Since the differences between the GRE and ERE (estrogen response element) can be found in the third and fourth base pairs of the two half-sites, it is conceivable that the three identified residues (or immediately adjacent amino acids) may actually contact these base pairs. Third, "missing base" contact and phosphate ethylation interference analyses show that the two DBD molecules interact with the major grooves of the GRE half-sites and that contacts are made with the corresponding phosphate groups located on the same face of the DNA (*27*).

A model of the DBD-GRE complex consistent with these observations is shown in Fig. 5. In this model, the α helix encompassing residues Ser^{459} to Glu^{469} is taken as a "recognition helix" located in the DNA major groove, with residues Gly^{458}, Ser^{459}, and Val^{462} in close spatial proximity to the two middle AT base pairs of the GRE half sites. With this location, Lys^{461}, Lys^{465}, and Arg^{466}, which are conserved within the family of glucocorticoid, estrogen, and androgen receptors, can contact other bases within the GRE or the DNA phosphate backbone. The size and shape of the DBD makes it possible to orient the two monomers to form protein-protein contacts involving the segment that is important for cooperativity. Further features of this model include several favorable electrostatic interactions between charged Arg, Lys, and His residues and the DNA phosphates. Finally, we note that point mutants at positions 488, 489, and 491 within the second finger bind normally to GRE sequences but fail to enhance transcription in vivo (*28*); these "positive control" defects have been interpreted to define a region of the receptor that may contact components of the transcription machinery, thereby regulating initiation. In our model, amino acids in this region of the second finger extend away from the DNA and so are potentially available to interact with other proteins.

We have presented a structure of the glucocorticoid receptor DBD and a model of the DBD-GRE complex that is consistent with experimental data. The DBD is folded in a single domain with several contacts between the two zinc finger regions, in contrast to the common view that the two zinc fingers should represent distinct subdomains. The structure of the steroid and thyroid hormone receptor fingers are significantly different from that of the TFIIIA type fingers (*29*). The large degree of sequence homology within the DBD between various members of the steroid and thyroid family of hormone receptors strongly suggests that the DBD structure of the other members of this family should be similar to the one we have presented.

Fig. 5. Model of the dimeric complex between the glucocorticoid receptor DBD and the glucocorticoid response element (GRE). The GRE consensus sequence is 5′-NNGGTACANNNTGTTCT-NN-3′. The DBD residues essential for discrimination between glucocorticoid and estrogen response elements (GRE and ERE) (*26*) as well as two AT base pairs in the center of each of the GRE half-sites, indicating the differences between this GRE and the consensus ERE sequences, are colored in red. DBD residues in the segment that are important for protein-protein interactions (*25*) are colored in green.

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7. The two protein fragments were KPARP(C440-G525)RL [numbering referring to the rat glucocorticoid receptor (*8*)], which was purified and checked for DNA-binding activity as described in (*2*), and S394-I519)RARYPGIL (numbering referring to the human glucocorticoid receptor), which was purified and checked for DNA-binding activity as described in (*3*). All sequence numbering in the text refers to the rat glucocorticoid receptor.
8. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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13. Sequential NOEs refer to neighboring residues. Medium and long-range NOEs are here defined as connectivities between amino acids up to four residues apart, and five or more residues apart, respectively.
14. NOESY spectra [J. Jeener, B. H. Meier, P. Bach-

mann, R. R. Ernst, *J. Chem. Phys.* 71, 4546 (1979); D. J. States, R. A. Haberkorn, D. J. Ruben, *J. Magn. Reson.* 48, 286 (1982)] were recorded at 300 K in 95:5 $^1H_2O/^2H_2O$ mixtures or 99.9% ^2H_2O in a buffer containing 300 mM NaCl, 0.1 mM NaN_3, and 1 mM phosphates at pH 6.5, with 1 mM DTE to prevent oxidation of cysteines. Spectra were recorded with mixing times of 10, 20, 40, and 80 ms ($^1H_2O/^2H_2O$) and 30, 60, 90, 120, and 150 ms (^2H_2O).
15. Buildup rates obtained in ^2H_2O were calibrated against Tyr2,6-Tyr3,5 NOEs (2.45 Å). Buildup rates in ^1H_2O involving NH protons were calibrated against the sequential NH_i-NH_{i+1} distance in α-helical regions [2.8 Å (*9*)]. Ten percent of the calculated distance, representing the experimental uncertainty, was added to the upper bound.
16. The pseudo-atom corrections were 1.0 Å for methylene and methyl groups, 1.5 Å for Leu dimethyl groups, and 2 Å for Phe and Tyr 3,5 and 2,6 protons.
17. Upper and lower distance bounds for the amide H and carbonyl O atoms were 2.3 and 1.85 Å, respectively.
18. Calculated for tetrahedral coordination assuming a Zn-S distance of 2.35 Å [L. Pauling, *The Chemical Bond* (Cornell Univ. Press, Ithaca, NY, 1967)].
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21. Values of the DG error function (*14*) obtained for tetrahedral coordination of the sulfurs of Cys^{476} and Cys^{482} and two of the Cys^{492}, Cys^{495}, and Cys^{500} sulfurs were 153 ± 56, 200 ± 77, and 90 ± 62 Å^4 after DG embedding and optimization, and 72 ± 51, 98 ± 60, and 36 ± 35 Å^4 after DDD, when excluding Cys^{492}, Cys^{495}, or Cys^{500}, respectively.
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23. DDD was run with a step size of 20 ps and a temperature coupling constant of 200 ps with a constraint force constant (*12*) of K_{dc} = 1 kJ mol^{-1} Å^{-4}.
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30. Supported by the Netherlands Organization for Chemical Research (SON) and the Netherlands Organization of Scientific Research (NWO); 600-MHz ^1H NMR spectra were recorded at the national Dutch HF-NMR facility in Nijmegen with the assistance of S. Wijmenga and J. Joordens. Additional financial support was obtained from the Swedish Medical Research Council (MFR), the Swedish National Board for Technical Development (to J.C.-D.), and NIH and NSF (grants to K.R.Y.). T.H. acknowledges a research fellowship from the Swedish National Research Council and financial support from the NWO. J.C.-D. acknowledges a research fellowship from MFR and K.D. acknowledges a predoctoral fellowship from MFR.

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Methyl Chloride Transferase: A Carbocation Route for Biosynthesis of Halometabolites

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Enzymatic synthesis of methyl halides through an *S*-adenosyl methionine transfer mechanism has been detected in cell extracts of *Phellinus promaceus* (a white rot fungus), *Endocladia muricata* (a marine red algae), and *Mesembryanthemum crystallium* (ice plant). This mechanism represents a novel pathway for the formation of halometabolites. The Michaelis constants for chloride and bromide ion and for *S*-adenosyl methionine in the reaction have been determined for the enzyme from *E. muricata*. A recent survey of marine algae indicates that there may be a broad distribution of this enzyme among marine algae.

THE MOST ABUNDANT HALOHYDROcarbon species in the upper atmosphere is methyl chloride, and it is widely believed that biological synthesis is largely responsible for sustaining a global emission rate estimated to be 5×10^6 tons of methyl chloride per year (*1*). The synthesis of methyl chloride by cultures of wood rot fungi has been well documented (*2*), and there have been isolated studies reporting the in vivo synthesis of methyl halides by marine macroalgae and phytoplankton (*3*). However, in vitro methyl chloride synthesis has not been reported. The established enzymatic mechanism for the biosynthesis of halometabolites involves the hydrogen peroxide–dependent oxidation of halides to form electrophilic halogenating species (*4*). The electrophilic halogen intermediate generated in the peroxidase-reaction can react with a broad spectrum of nucleophilic acceptors to form the halometabolites. In our laboratory we demonstrated the synthesis of one prevalent atmospheric halohydrocarbon, methyltribromide, through the peroxidatic route (*5*). In the synthesis of this compound, bromoperoxidase first catalyzes the multiple bromination of an activated methylene carbon atom adjacent to a ketone function (*6*). The enzymatic bromination reaction is followed by the nonenzymatic hydrolysis of the tribrominated methyl group to release methyltribromide in a classical bromoform reaction. However, all of our attempts to detect monohalomethanes through chloro- or bromoperoxidase-type reactions have been unsuccessful. Thus we have recently turned our attention to a potential alternative route for the synthesis of methyl halides. White (*7*) has shown that when the fungus *Phellinus pomaceus* is grown on deuterium-labeled glucose, serine, or methionine, the methyl chloride produced is also labeled. This observation is consistent with the methyl chloride being derived from methionine. A likely route for this reaction would be through the methyl donor, *S*-adenosyl methionine. We report the detection and partial purification of a methyl transferase that catalyzes the methylation of chloride, bromide, and iodide ions.

Methyl chloride transferase activity has been detected in cell-free extracts prepared from the fungus *P. pomaceus*, from the ma-

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Table 1. Methyl transferase activity levels in whole cells and crude cell extracts. The activity measurements for fungal cells were obtained by growing cultures in 100 ml of 5% malt extract, 100 mM KCl, 1% bactopeptone liquid media in sealed bottles. Gas samples were removed each day and analyzed as described in Fig. 1. Known amounts of methyl chloride in identically prepared bottles were used as standards. The value reported here was the amount of methyl chloride obtained during peak production periods. The values for whole cells of *E. muricata* and *M. crystallium* were obtained by incubating whole cells in 100 mM KCl in gas-tight vials. Gas samples were analyzed as in Fig. 1 with known amounts of methyl chloride as standards. The values for all crude cell extracts were obtained by incubating the cell extracts in 4-ml reaction mixtures containing 250 mM KCl, 500 μM SAM, 100 mM phosphate, pH 6.8. The *P. pomaceus* cell extracts were obtained by digesting cells with 10 mg of Novozyme 234 per milliliter for 1 hour followed by removal of cell debris by centrifugation. Cell extracts of *E. muricata* were obtained as in Fig. 1. Cell extracts of *M. crystallium* were obtained by grinding the cells in a Waring blender. Gas samples were analyzed in the same manner as those from whole cells.

Source	Methyl chloride production	
	Whole cells (pmol g^{-1} day^{-1})	Crude extract (fmol min^{-1} mg^{-1})
P. pomaceus	7	25
E. muricata	30	670
M. crystallium	19	3

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A New Function for the C-terminal Zinc Finger of the Glucocorticoid Receptor

REPRESSION OF RelA TRANSACTIVATION*

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Glucocorticoids inhibit NF-κB signaling by interfering with the NF-κB transcription factor RelA. Previous studies have identified the DNA-binding domain (DBD) in the glucocorticoid receptor (GR) as the major region responsible for this repressive activity. Using GR mutants with chimeric DBDs the repressive function was found to be located in the C-terminal zinc finger. As predicted from these results the mineralocorticoid receptor that contains a C-terminal zinc finger identical to that of the GR was also able to repress RelA-dependent transcription. Mutation of a conserved arginine or a lysine in the second zinc finger of the GR DBD (Arg-488 or Lys-490 in the rat GR) abolished the ability of GR to inhibit RelA activity. In contrast, C-terminal zinc finger GR mutants with mutations in the dimerization box or mutations necessary for full transcriptional GR activity were still able to repress RelA-dependent transcription. In addition, we found that the steroid analog ZK98299 known to induce GR transrepression of AP-1 had no inhibitory effect on RelA activity. In summary, these results demonstrate that the inhibition of NF-κB by glucocorticoids involves two critical amino acids in the C-terminal zinc finger of the GR. Furthermore, the results from the use of mineralocorticoid receptor and anti-glucocorticoids suggest that the mechanisms for GR-mediated repression of NF-κB and AP-1 are different.

Glucocorticoid hormones regulate many different biological processes via a specific intracellular receptor, the glucocorticoid receptor (GR),[1] which is present in most cell types. The GR is a member of the superfamily of nuclear receptors, which all contain three main functional domains (1, 2). After binding of hormone to the C-terminal ligand binding domain (LBD) and dissociation of heat shock proteins, the GR homodimerizes and interacts with specific DNA sequences termed glucocorticoid response elements (GREs) through its central DNA binding domain (DBD). The transcriptional activity of GR is mainly dependent on the τ1 domain localized in the N-terminal part of the protein (3, 4). The highly conserved DBD contains two zinc fingers and in each of them a zinc ion is tetrahedrally coordinating four cysteine residues. One function of the DBD is to discriminate between different response elements, thus determining target genes to be activated (5, 6). This function is achieved by a few amino acids localized in the C-terminal part of the N-terminal zinc finger, the so-called the P box. A second subdomain termed the D box in the N-terminal knuckle of the C-terminal zinc finger has been shown to harbor amino acid residues important for homodimerization (7). DNA binding of the ligand-activated GR results in an increased rate of formation of transcriptionally competent pre-initiation complexes. This is thought to be achieved by protein-protein interactions between the receptors and different components of the transcriptional machinery (8, 9). Besides the more well studied transcriptional activation process, the GR can repress transcription via different mechanisms (10). The GR has been shown to bind to overlapping DNA response elements for other transcription factors leading to repression (11–13). These GR binding elements have been termed negative GREs. Inhibition of gene expression by glucocorticoids can also occur in the absence of GR DNA binding. The most well studied system for this is the repression by the GR of genes activated by the AP-1 transcription factor complex. In this case there is evidence for a direct physical association between the proteins present in AP-1 and the GR (14–16). Although direct binding of the GR to DNA is not necessary, the DBD has in some cases been shown to be essential for this interaction (15). Furthermore, the composition of the AP-1 complex determines whether the GR will cause a positive or a negative effect on AP-1 controlled transcription. In addition to the ability of GR to interfere with AP-1 controlled transcription, we and other (17–20) have shown that the GR also can repress NF-κB signaling.

NF-κB is an inducible transcription factor complex that plays an essential role in the inflammatory and immune responses (21). It is activated by a diverse range of signals including the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1 as well as phorbol esters, physical or oxidative stress, and bacterial and viral proteins. In most cells the NF-κB is composed of a heterodimer between RelA (p65) and NFKB1 (p50), where the RelA protein is responsible for the transactivation potential. In the non-activated state the NF-κB is sequestered in the cytoplasm through the interaction with the inhibitory protein IκB. During activation, the IκB protein becomes phosphorylated and degrades allowing NF-κB to translocate to the nucleus where it binds to specific DNA elements and subsequently regulates genes involved in inflammation and immune responses (22–24).

Glucocorticoids have potent immunosuppressive effects and are commonly used in the clinic to suppress different immunological and inflammatory responses. Different molecular mech-

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[1] The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid response element; AR, androgen receptor; DBD, DNA binding domain; LUC, luciferase; MR, mineralocorticoid receptor; PR, progestin receptor; RARα, retinoic acid receptor α; TRβ, thyroid receptor β; PCR, polymerase chain reaction.

anisms have been suggested to be involved in this process including inhibition of AP-1 and NF-κB (25). In an earlier report (19) using transient transfections of GR and NF-κB responsive reporter genes, we have shown a mutual transcriptional inactivation between the GR and the NF-κB protein RelA. Direct DNA binding of GR is not required for the NF-κB repression to occur, since a GR, in which the P box had been mutated so that it no longer recognized a GRE, still was able to repress. Instead we showed that the GR and the RelA can directly or indirectly interact with each other *in vitro* and mutually interfere with transcriptional activity. Using deletion mutants and chimeric receptors, we demonstrated that the GR DBD is the major GR domain responsible for repression of RelA activity. In addition, an alternative mechanism for glucocorticoid repression of NF-κB activity has been suggested, which involves induction of IκBα by GR leading to the sequestering of NF-κB in the cytoplasm (26, 27).

In this report we investigated the subdomain and critical amino acids in the GR DBD involved in glucocorticoid repression of the NF-κB protein RelA. In addition, we investigated the role of GR dimerization and transactivation for the repression of RelA activity. Finally, we analyzed the ability of glucocorticoid antagonists to cause repression of NF-κB activity.

EXPERIMENTAL PROCEDURES

Reagents—Deep Vent® DNA polymerase and T4 DNA ligase were from New England Biolabs. Media, antibiotics, fetal bovine serum, and Lipofectin® were purchased from Life Technologies, Inc. Dexamethasone and aldosterone was obtained from Sigma. The monoclonal antibody number 7 against rat GR has previously been described (28). RU486 was obtained from Roussel-UCLAF (Romainville, France) and ZK98299 was from Schering, Berlin, Germany.

Reporter and Expression Plasmids—The luciferase reporter plasmids 3xNF-κB(IC)tk-LUC and $(GRE)_2$tk-LUC (29, 30) and the expression plasmid RcCMV-RelA (19) have been described previously. GR mutants with chimeric DBDs were constructed using the PCR-ligation-PCR protocol (31). For this purpose the DBDs of GR and thyroid receptor β (TRβ) were separated into three parts as follows: the N-terminal zinc finger, the linker region between the two fingers, and the C-terminal zinc finger, and termed ggg or ttt, respectively (the first, second, and third lowercase letter representing the N-terminal zinc finger, linker region, and C-terminal zinc finger region of GR (g) or TRβ (t), respectively). The expression plasmids GR_{nx} (32) and GTG (33) in this report named GgggG and GtttG, respectively, were used for the amplification of the different DBD regions. The first PCR reaction contained 1 ng of plasmid DNA, 250 μM each dNTP, 1 μM primers, 1 × reaction buffer, and 1 unit of Deep Vent® DNA polymerase. The PCR program contained a 30-s denaturation step at 94 °C, 30 s annealing time at 56 °C, and a 30-s extension at 72 °C, for 25 cycles. Plasmid DNA templates and pairs of primers were as follows: PCR-1, GgggG, 5′-AAGCCCCAGCATGAGACCAGAT-3′ (primer A) and 5′-GCAGCCTTCACACGTGATACAG-3′; PCR-2, GgggG, primer A and 5′-ACATAGGTAATTGTGCTGTCCTTCC-3′; PCR-3, GgggG, 5′-GCTGGAAGGAATGATTGCATC-3′ and 5′-ACTCCTGTAGTGGCCTGCTGAA-3′ (primer B); PCR-4, GtttG, 5′-AAGGGTTTCTTTAGAAGGACCATTC-3′ and 5′-TACCAGGATTTTCAGAG-GTTTC-3′; PCR-5, GtttG, 5′-AAATATGAAGGAAAATGTGTCATAGACA-3′ and primer B; PCR-6, GtttG, primer A and 5′-ACAGGAATAGGATGGATGGAGATT-3′. After phosphorylation, 5 μl of PCR-1, -2, and -3 were mixed with 5 μl of PCR-4, -5, and -6, respectively, and ligated with 400 units of T4 DNA ligase for 15 min at room temperature. The resulting fragments were amplified in a second PCR reaction using primers A and B and under the same conditions as for the first PCR reaction. The PCR products were digested with *Not*I and *Xho*I and inserted into the GR_{nx} expression plasmid instead of the wild type GR DBD to create the chimeric GR/TRβ-DBD mutants GgttG, GggtG, and GttgG. The GgtgG mutant was constructed by using the GgttG expression plasmid as template DNA instead of the GgggG in the PCR-6. The rat GR mutants R488Q, K490E, and N491A were created by digesting the corresponding yeast expression vectors (34) with *Nco*I and *Pst*I, and the isolated GR fragments containing the mutation were subcloned into the corresponding sites of the mammalian rat GR expression vector 6RGR (35) after removing the wild type sequence. All constructs were verified by sequencing.

Cell Culture and Transfections—Green monkey COS-1 cells were grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 7.5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Cells were plated in 24-well multidishes at a density of 3×10^4 cells/well 24 h before transfection. Cells were transiently transfected using Lipofectin® according to the recommendations of the manufacturer. In transrepression experiments, cells were co-transfected with 200 ng of 3xNF-κB(IC)tk-LUC reporter plasmid, 2.5 ng of RelA expression plasmid, and 25 ng of wild type or mutant GR expression plasmid. In the transactivation experiments, cells were co-transfected with 200 ng of $(GRE)_2$tk-LUC and 25 ng of wild type or mutant GR expression plasmid. Following overnight exposure of the cells to the DNA/lipid mixture, fresh medium was added, and cells were incubated in the absence or presence of 1 μM dexamethasone for 24 h. Cells were lysed and luciferase activity was determined.

Western Blotting—COS-1 cells were plated in 10-cm cell culture dishes and transfected with 1 μg of expression plasmids for wild type or mutant GR as described above. Cells were pelleted, resuspended in 200 μl of ETG buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 10% (v/v) glycerol) containing 0.4 M KCl, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 mM dithiothreitol and homogenized with a micro-Dounce homogenizer by 20 strokes. The homogenates were centrifuged at 265,000 × *g* for 40 min at 4 °C. Protein concentrations were determined, and supernatants were mixed with 1 volume of 2 × SDS buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% (v/v) glycerol), and 10 μg of total protein was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to a C-Extra Hybond membrane (Amersham Corp.). Immunodetection was carried out using the enhanced chemiluminescence detection kit from Amersham Corp. after incubation with the monoclonal antibody number 7 (28) followed by secondary horseradish peroxidase-labeled anti-mouse antibody (Amersham Corp.) according to the suggested protocols.

RESULTS

The C-terminal Zinc Finger of the GR DBD Is Responsible for the Interference with RelA Transactivation—We and others (19, 20) have previously shown that the DBD of the GR is of crucial importance for the ability of GR to interfere with the NF-κB-mediated response, since deletion of the GR DBD or replacement of the GR DBD with the corresponding TRβ DBD abolished repression. To further determine if a particular region in the GR DBD is responsible for the functional interference with NF-κB activity, GR mutants were created in which individual parts of the DBD were replaced by the corresponding regions of the TRβ DBD. For this purpose the GR DBD was divided into three parts, the N-terminal zinc finger, the linker region, and the C-terminal zinc finger, respectively (Fig. 1*A*). The ability of the different GR mutants to repress NF-κB was tested on RelA-mediated transactivation of a luciferase reporter gene controlled by three NF-κB sites from the human intercellular adhesion molecule-1 promoter and a minimal thymidine kinase promoter (3xNF-κB(IC)tk-LUC) in COS-1 cells. Exchanging the whole GR DBD with the DBD from TRβ (GtttG) resulted in a 70–80% loss of repressive activity in comparison to the wild type GR (GgggG) (Fig. 1*B*). Exchanging the C-terminal zinc finger alone (GggtG) or the linker region together with the C-terminal zinc finger (GgttG) with the corresponding region of TRβ DBD destroyed the ability of the GR to inhibit RelA-mediated transactivation to a similar degree as for GtttG (Fig. 1*B*). In contrast, exchanging the linker region alone (GgtgG) did not affect the repressive activity of GR. This suggested that this region was dispensable and that the C-terminal zinc finger was critical for the repression to occur. The importance of the C-terminal zinc finger was confirmed with the GttgG mutant, since most of the repressive activity was maintained when the C-terminal zinc finger alone was from the GR (Fig. 1*B*). These results demonstrate that the contribution of the GR DBD to the functional interference between GR and RelA is localized in the C-terminal zinc finger of the GR.

Since the amino acid sequences of the C-terminal zinc finger of the GR and the mineralocorticoid receptor (MR) are identical

(36), this would suggest that the MR is able to repress RelA-mediated transactivation. Indeed, in transfection experiments performed as above, the MR activated by 10 nM aldosterone repressed RelA-mediated transactivation as efficiently as the GR (data not shown). This also shows that the four amino acids outside the second zinc finger that differ between the MR and GR DBDs are not critical for the repressive capacity.

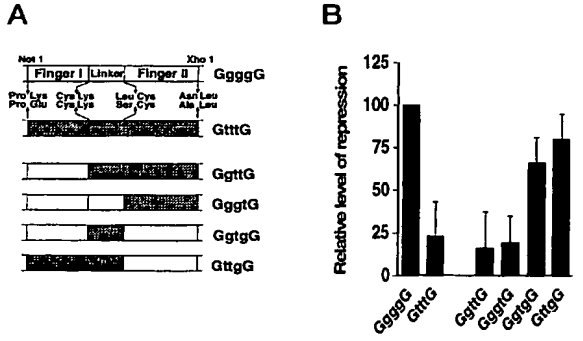

FIG. 1. **Importance of the GR DBD C-terminal zinc finger for repression of RelA-mediated transactivation.** *A*, schematic representation of the DNA binding domains of the different GR mutants with chimeric GR/TRβ DBDs used for identifying regions in the GR DBD important for repression of RelA activity. *B*, COS-1 cells were transiently transfected with the 3xNF-κB(IC)tk-LUC reporter plasmid together with expression plasmids coding for RelA and the various GR/TRβ DBD mutants. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The wild type GR (GgggG) repressed RelA activity by 50–60% in the presence of hormone as compared with the luciferase activity in the absence of hormonal treatment. This level of repression was given the nominal value of 100, and repression by the GR mutants was expressed relative to this nominal value. Values are mean ± S.D. from three independent experiments.

An Intact GR Dimerization Box Is Not Required for Repression of RelA-mediated Transactivation—A major function for the C-terminal zinc finger in the GR DBD is to contribute to receptor homodimerization, a prerequisite for the receptor to bind DNA and transactivate efficiently (7, 37). This function is achieved by the D box region which is localized in the N-terminal knuckle of the C-terminal zinc finger. Since dimerization and NF-κB repression functions are localized in the same zinc finger of the GR, we tested if GR dimerization is a prerequisite for repression of Rel A transactivation. For this purpose we used a GR mutant (D4X) in which three amino acids out of five in the D box have been mutated (Fig. 2*A*) (38). Transfection experiments with the D4X mutant demonstrated that mutations in the D box did not impair the ability of GR to repress RelA transcriptional activity (Fig. 2*B*). This shows that receptor dimerization is not a prerequisite for GR-mediated repression of RelA. As previously shown, the D4X mutant harbored no significant transcriptional activity (Fig. 2*C*).

Identification of GR Amino Acids Involved in Repression of RelA-mediated Transactivation—Previous studies have shown that in addition to GR, the estrogen receptor, progestin receptor (PR), and the androgen receptor (AR) also repress NF-κB activity (39–41). In addition, as shown in this study, the MR also has this capacity. In contrast, the TRβ, the retinoic acid receptor α isoform (RARα), and the ecdysone receptor are un-

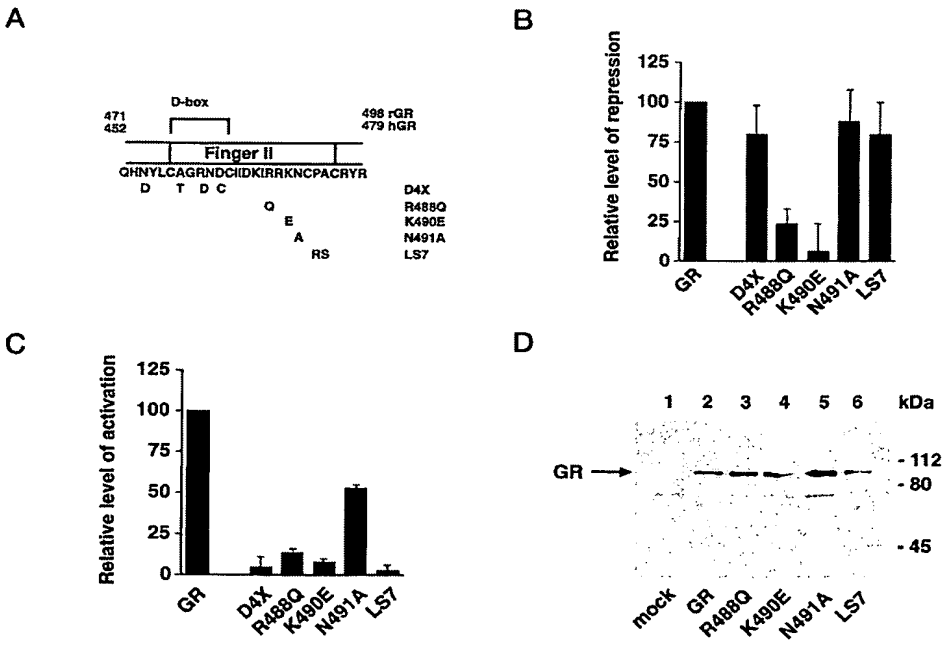

FIG. 2. **GR amino acids involved in repression of RelA-mediated transactivation.** *A*, schematic representation of the tip of the C-terminal zinc fingers from the different GR mutants used showing the amino acids that were mutated to identify the critical residues for RelA repression. *B*, COS-1 cells were transiently transfected with the 3xNF-κB(IC)tk-LUC reporter plasmid together with expression plasmids coding for RelA and the various GRs with mutations in the C-terminal zinc finger. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The wild type GR (GgggG) repressed RelA activity by 50–60% in the presence of hormone as compared with the luciferase activity in the absence of hormonal treatment. This level of repression was given the nominal value of 100, and repression by the GR mutants was expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments. *C*, COS-1 cells were transiently transfected with the $(GRE)_2$tk-LUC reporter plasmid together with expression plasmids coding for the various GR mutants. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The induction factor for the wild type GR (GgggG) was given the nominal value of 100, and the induction levels of the GR mutants were expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments. *D*, expression levels for the wild type and mutant GRs were determined by Western blotting after transfection into COS-1 cells.

TABLE I
Comparison of the amino acid sequences in the tips of the second zinc finger of RelA repressive and some non-RelA repressive nuclear receptors

The amino acid sequences for receptors other than the GR are only given if they differ from the GR sequence. The two bold amino acids in the GR sequence indicate amino acids conserved only among the RelA repressive receptors. EcdR, ecdysone receptor; ER, estrogen receptor.

Receptors	C-terminal zinc finger tip	RelA repression
GR	CIIDKIRRKNC	Yes
MR		Yes
PR	V	Yes
AR	T F	Yes
ER	N S	Yes
RARα	N VT NR	No
TRβ	V VT NQ	No
EcdR	EMNMYM RK	No

able to repress RelA-mediated transactivation (19).[2] An amino acid sequence comparison of the C-terminal zinc fingers of repressive and non-repressive receptors revealed that the arginine and the lysine in position 488 and 490 in the rat GR are conserved only in the repressing receptors, suggesting an important role for these amino acids in the repressive activity (Table I). To test the importance of these amino acids for GR-mediated repression of RelA activity, we exchanged these amino acids in the rat GR to a glutamine and a glutamic acid, respectively (R488Q and K490E, respectively), and tested the ability of the mutated GRs for their ability to repress RelA transcriptional activity in COS-1 cells. Transfection experiments showed that both GR mutants, R488Q and K490E, had lost almost all their repressive activity as compared with the wild type GR (Fig. 2*B*). In contrast, the GR mutants N491A and LS7 with substitutions of amino acids that are not conserved among the repressive receptors (Fig. 2*A*, Table I) had retained their ability to repress RelA activity. None of these C-terminal zinc finger GR mutants except the N491A mutant could transactivate the $(GRE)_2$tk-LUC reporter gene. The N491A mutant retained approximately 50% transcriptional activity as compared with the wild type GR (Fig. 2*C*). The inability of the R488Q and K490E mutant to repress the RelA activity was not due to poor expression of the receptor proteins, since Western blot analysis of the transfected cells showed that the expression levels of the mutated receptors were the same as for the wild type GR (Fig. 2*D*). These results demonstrate that the arginine and the lysine residues in positions 488 and 490 in the rat GR (corresponding to positions 469 and 471 in the human GR) are critical for GR-mediated inhibition of RelA-dependent transactivation.

Different Ability of Glucocorticoid Antagonists to Promote GR-dependent Repression of RelA-mediated Transactivation—The steroid analogs RU486 and ZK98299 are antagonists of GR transactivation but are able to stimulate GR transrepression of AP-1 activity (38). Furthermore, we and others (19, 20, 40) have previously shown that RU486 can also act as a partial agonist for GR- and PR-mediated repression of NF-κB activity. We tested if the steroid analog ZK98299 could inhibit NF-κB activity in analogy to RU486. As shown in Fig. 3*A*, 10 nM RU486 repressed RelA activity to a level that was approximately 40% the repression obtained with 10 nM dexamethasone. In contrast, no repression was observed with 10 or 100 nM ZK98299. This was not due to the lack of biological activity of ZK98299, since it could inhibit GR transactivation as efficiently as RU486 (Fig. 3*B*). These results suggest that RU486

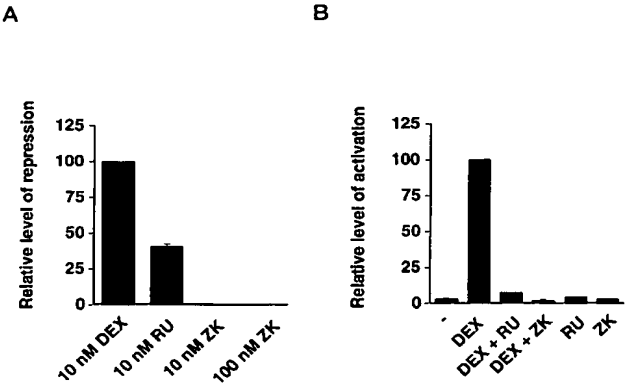

FIG. 3. **Comparison of dexamethasone, RU486, and ZK98299 for repression of RelA-mediated transactivation.** *A*, COS-1 cells were transiently transfected with the 3xNF-κB(IC)tk-LUC reporter plasmid together with expression plasmids coding for RelA and the wild type GR. After transfection the cells were treated with 10 nM dexamethasone (*DEX*), 10 nM RU486 (*RU*), 10 and 100 nM ZK98299 (*ZK*) as indicated in the figure. After 24 h of treatment luciferase activity was determined. The repression obtained with 10 nM dexamethasone was given the nominal value of 100, and the repression for the other treatments was expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments. *B*, COS-1 cells were transiently transfected with the $(GRE)_2$tk-LUC reporter plasmid together with expression plasmid coding for the wild type GR. After transfection the cells were not treated (−), treated with 10 nM dexamethasone, 100 nM RU486, 100 nM ZK98299, or combinations of 10 nM dexamethasone and 100 nM RU486 (*DEX + RU*) or 10 nM dexamethasone and 100 nM ZK98299 (*DEX + ZK*). After 24 h of treatment luciferase activity was determined. The induction obtained with 10 nM dexamethasone was given the nominal value of 100 and the induction for the other treatments was expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments.

and ZK98299 induce GR to states with different competence to repress RelA activity.

DISCUSSION

The recent discovery of an inhibitory cross-talk between the NF-κB and GR signaling pathways has provided one molecular mechanism by which glucocorticoids exert their potent anti-inflammatory effects (17, 19, 20). The inhibition of NF-κB activity by estrogen receptor, PR, and AR has also been reported (39–42). Here we show that the MR also has the ability to repress RelA activity in a co-transfection assay. Thus, all steroid receptors are able to repress NF-κB in contrast to nuclear receptors from the RAR/TR subfamily, suggesting that a distinct structural determinant within the steroid receptor subfamily is responsible for the repression of NF-κB. The importance of the DBD in these receptors for the repressive activity has been demonstrated in several previous studies, where it was shown that deletion or replacement of the whole DBD resulted in the loss of the repressive activity (19, 20, 39–41). To identify which subdomain in the GR DBD is responsible for the repression of RelA activity, we have used GR mutants in which various parts of the GR DBD have been replaced with the corresponding regions of the non-repressive TRβ DBD. Our results demonstrate that most of the repression of NF-κB activity could be attributed to the C-terminal zinc finger of the GR DBD. This localizes a new function to this finger, which previously has been known mainly to harbor functions important for dimerization and transactivation (7, 37). Analysis of two C-terminal zinc finger GR mutants with substitutions of the arginine (amino acids 488 in the rat GR) and lysine (amino acids 490 in the rat GR) to a glutamine and a glutamic acid, respectively, confirmed the importance of this finger and identified two critical basic residues for repression of NF-κB activ-

[2] J. Liden, unpublished observations.

ity. No particular function has previously been attributed to the arginine residue 488 (corresponding to amino acid 469 in the human GR) with regard to dimerization or interaction with DNA, since substitution of this residue to a glutamine did not impair DNA binding (34). The lysine residue 490 (corresponding to amino acid 471 in the human GR), on the other hand, is involved in making contact with the phosphate backbone (43). Interestingly, these two residues are conserved only in the C-terminal zinc finger of steroid receptors, consistent with the observation that only members of this subfamily of nuclear receptors seem to be able to repress NF-κB. Mutation of either of these two amino acids resulted in a significantly decreased transcriptional activity of GR (this study and Ref. 34). However, no correlation between transcriptional activation by GR and transrepression of RelA activity exists, since the two GR mutants D4X and LS7, which lack most of their transcriptional activity, were fully active with regard to repression of RelA activity. This is also in line with previous data showing that GR mutants with a deletion of the major transactivation domain τ1 or a substitution of the P box by that of the TRβ were still repressive (19, 20). Thus, glucocorticoid induction of the NF-κB inhibitor IκBα which previously has been suggested as a mechanism controlling NF-κB inhibition by glucocorticoids cannot explain why transcriptionally deficient GR mutants can still repress NF-κB (26, 27). In addition, it has been shown in monocytic U937 cells that inhibition of ICAM-1 gene expression by glucocorticoids occurs in the absence of protein synthesis (44). More recently, several studies also reported that NF-κB could be inhibited in osteoblast U2-OS cells (39), alveolar epithelium-like A549/8 cells (45), kidney epithelial NRK-52E cells (46), and aortic endothelial BAEC cells (47) in the absence of IκBα induction. Collectively, these data argue for a general mechanism for glucocorticoid inhibition of NF-κB which involves GR transrepression via protein-protein interaction between non-DNA binding GR and NF-κB transcription factors as initially suggested (17, 19, 20). However, induction of IκBα may play a more significant role in some specific cell types such as lymphocytes (47).

Several results reported here together with previous observations indicate that the mechanisms by which GR inhibits NF-κB and AP-1 signaling pathways are different. Most notable is that MR, as reported in this study, is an efficient inhibitor of NF-κB, whereas it is known to be a very weak repressor of AP-1 activity (48, 49). In contrast, RARα is unable to repress NF-κB activity but is able to inhibit AP-1 (19, 50). These differences may be related to the fact that although the GR DBD is critical for repression of both NF-κB and AP-1, different parts of the DBD contribute to this effect. This is supported by the data from Heck *et al.* (38) who demonstrated that point mutations within the N-terminal zinc finger of GR could severely impair AP-1 repression, whereas our results indicate that this zinc finger does not play a significant role in the inhibition of NF-κB activity. In addition, previous data showed that GR mutants with deletion of the N-terminal domain could efficiently inhibit NF-κB but not AP-1 (15, 19, 20). It has recently been suggested that the mechanism by which GR represses AP-1 involves competition between GR and AP-1 for limiting amounts of the co-activator CBP/p300 (51). Although, as recently reported, a physical interaction between CBP/p300 and RelA also occurs (52, 53), no evidence has yet been reported that GR inhibits NF-κB by competing for the co-activator CBP/p300. However, if this occurs, the mechanism is likely to be different from that of the GR and AP-1 cross-talk, since some receptors such as RAR for instance can interact with CBP/p300 and inhibit AP-1 but have no repressive activity on NF-κB (51). Finally, by using glucocorticoid antagonists, we also found that ZK98299 is unable to repress NF-κB activity, whereas it has previously been shown that the ZK98299·GR complex is an efficient inhibitor of AP-1 activity (38). This shows that ligands can exhibit a selectivity with regard to GR transrepression, probably as a consequence of different receptor conformations required for inhibiting various signaling pathways.

In conclusion, we have identified two critical residues within the C-terminal zinc finger of the GR DBD that are critical for repression of NF-κB activity. This finding identifies a new function for the GR C-terminal zinc finger. Furthermore, using C-terminal zinc finger GR mutants and antagonists, we further characterized the mechanism by which GR represses NF-κB, and we obtained evidence that the mechanisms by which GR inhibits NF-κB and AP-1 have different features. This knowledge could be very useful in the search for new GR ligands with selective activity for GR transrepression of different signaling pathways. Such ligands could be a useful tool for basic research regarding mechanisms of glucocorticoid action and possibly in clinical use with less side effects.

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